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THE DEVELOPMENT OF CUTANEOUS AND ORAL PIGMENTATION IN LABRADOR RETRIEVER FETUSES (*CANIS FAMILIARIS*)*

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ABSTRACT

Microscopic features of melanoblasts and melanocytes were studied in skin and oral mucosa of Labrador retriever fetuses (*Canis familiaris*).

Earliest melanoblasts were demonstrable only after treatment with ammoniacal silver nitrate (ASN) in the primordial dermis of the head, thorax, and the abdomen of 25 to 28 mm fetuses. The melanoblasts were most numerous in the lower two-thirds of the primordial dermis in contact with or near blood vessels. The frequent contact or close association of melanoblasts with blood vessels suggests that the cells migrate along blood vessels in their journey from the neural crest to their destination in the epidermis.

Dendritic dermal melanocytes first appear in the 58 mm fetus.

Earliest epidermal melanocytes were demonstrated in vertical sections and full thickness skin "spreads" of untreated, dopa-treated and ASN-treated tissue. Only scattered epidermal melanocytes were demonstrated in 25 to 28 mm fetuses. Their numerical distribution in the various regions of the body appeared to conform to a dorsoventral gradient.

Melanocytes were observed in primordial and differentiated hair follicles, eccrine sweat glands, and sebaceous glands.

This report was derived from an investigation related to the identification and histochemical features of melanoblasts and melanocytes in skin and oral mucosa of Labrador retriever fetuses (*Canis familiaris*). A subsequent report will present the fine structure of canine fetal melanocytes.

Specific objectives of the investigation were:

1. To determine the earliest stage of gestation in which melanoblasts and melanocytes could be identified with certainty and to record subsequent alterations in their structure and staining reactions.
2. To determine whether melanocytes appear in hair follicles after or during formation of the follicle.
3. To determine whether or not melanocytes were present in the fetal dermis at or near the time of birth.

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Experimental evidence (1–5) supports the view that cutaneous melanocytes originate as melanoblasts from the neural crest. Melanoblasts migrate to locations where they differentiate to melanocytes. While the migration pathways are not definitely known, DuShane (6) suggested that melanoblasts migrate ventrolaterally, beneath the epidermis. Detweiler (7) reported that trunk neural crest cells migrate between the ectoderm and the neural tube.

Zimmermann and Cornbleet (8) and Becker and Zimmermann (9) identified melanocytes in the epidermis of Negro fetuses early in the third month of gestation. Approximately one month later, melanocytes were also present in the epidermis of trunk regions. Early attempts to identify melanoblasts in the dermis of human fetuses failed. Zimmermann and Becker (10) later identified melanoblasts in the dermis of Negro fetuses during the tenth week of gestation after treatment with ammoniacal silver nitrate (ASN).

There is a paucity of information on the histologic features of primordial and differentiated skin of prenatal dogs; curiously, information about the skin of postnatal dogs also is limited. Schwartzman and Orkin (11) found

that melanocytes were numerous in the skin of foot pads and the planum nasale; the cells did not increase in number from birth to seven months. Szabó (12) utilized the dopa reaction on epidermal sheets of normal adult canine skin to demonstrate epidermal melanocytes in foot pads, tail, external ear, planum nasale, and forehead; melanocytes were not observed in similar dermal preparations. Baker (13) did not find pigment cells in the dermis of newborn dogs.

MATERIALS AND METHODS

Fetuses were removed from bitches 18, 20, 25, 28, 29, 30, 32, 33, 37, 40, 46, and 55 days after breeding (AB). Their size, weight, and development of the integument were recorded.

Twenty-nine embryos and fetuses obtained from five black Labrador retriever bitches were utilized in the study. The fetuses were procured either by excising the entire uterus or by surgical removal of 1 or 2 embryos from the intact uterus. The latter procedure yielded as many as 6 embryos of 5 different ages from the same bitch. The surgical procedure used was similar to one described by Evans (14).

Whole embryos and tissues were fixed in 10% buffered formalin. Embryos from 5 mm to 40 mm in length were processed and embedded whole or quartered. Full thickness skin specimens were obtained from the abdomen, back, eyelid, external ear, feet, lip, neck, tail, planum nasale, scalp, and oral mucosa of fetuses more than 40 mm in length.

Serial or representative sections of whole embryos and selected skin sites were cut with a microtome at 6 μ and stained by several methods including Harris's alum hematoxylin and eosin, periodic acid-Schiff reaction, toluidine blue, Masson's ammoniacal (reduced) silver nitrate (15, 16) and the paraffin section method for the dihydroxyphenylalanine (dopa) reaction (17).

Full thickness primordial and differentiated skin specimens from 8 fetuses were dehydrated, cleared, and mounted flat to permit observation of the spatial arrangement and structure of melanocytes. The tissues were either treated with 0.001% dopa solution or with 10% ammoniacal silver nitrate solution or were processed and mounted unstained.

RESULTS

Macroscopic Observations

The 29 fetuses ranged in weight from less than a gram to 300 grams and had crown-rump lengths from 5 to 195 mm (Table I).

Skin pigment was not visible in fetuses of less than 45 mm. Retinal pigment was first detected in a 13 mm embryo (Fig. 1), while skin pigment was first observed in a 58 mm fetus (Fig. 2).

TABLE I

Biometrics of black Labrador retriever fetuses

Identification	Crown-rump length (mm)*	Weight (gm)*	Days after breeding ±1
A1	25	1.4	29
A2	28	1.7	29
A3–5	98	42.4	40
B1–8	6–7	—	20
C1	5	—	18
C2-3	13	—	25
C4	19	—	28
D1	32	2.3	30
D2	36	4.2	32
D3	42	4.2	32
D4	58	13.2	37
D5	160	137.0	46
D6	195	300.0	55
E1-6	42–45	4.4	33

* Lengths and weights were determined on fresh specimens.

— = Data not obtained.

In 98 mm fetuses (Fig. 3) cutaneous pigmentation was prominent on the muzzle, eyelids and ears.

A fetus of 160 mm (Fig. 4) was heavily pigmented except for parts of the digital pads, the central part of the planum nasale, the metapodial pads, median upper and lower lip, chin, hard palate and tongue. Skin pigmentation was most prominent on the dorsal and lateral aspects of the head and body. A nearly full term fetus of 195 mm had abundant hair and was completely pigmented externally including digital pads and claws although the claws were somewhat less pigmented than the integument. The oral cavity was not pigmented except for the lips.

Microscopic Findings

1. *Melanoblasts and melanocytes in fetal dermis.* Melanoblasts were first demonstrated in the mesenchyme of 25 to 28 mm fetuses after the tissue was treated with ASN solution (Fig. 5). Silver impregnated melanoblasts were round to ovoid cells and averaged 5.23 ± 1.05* μ x 8.65 ± 2.77 μ. They were observed in the developing dermis of the head, especially near

* Standard deviation.

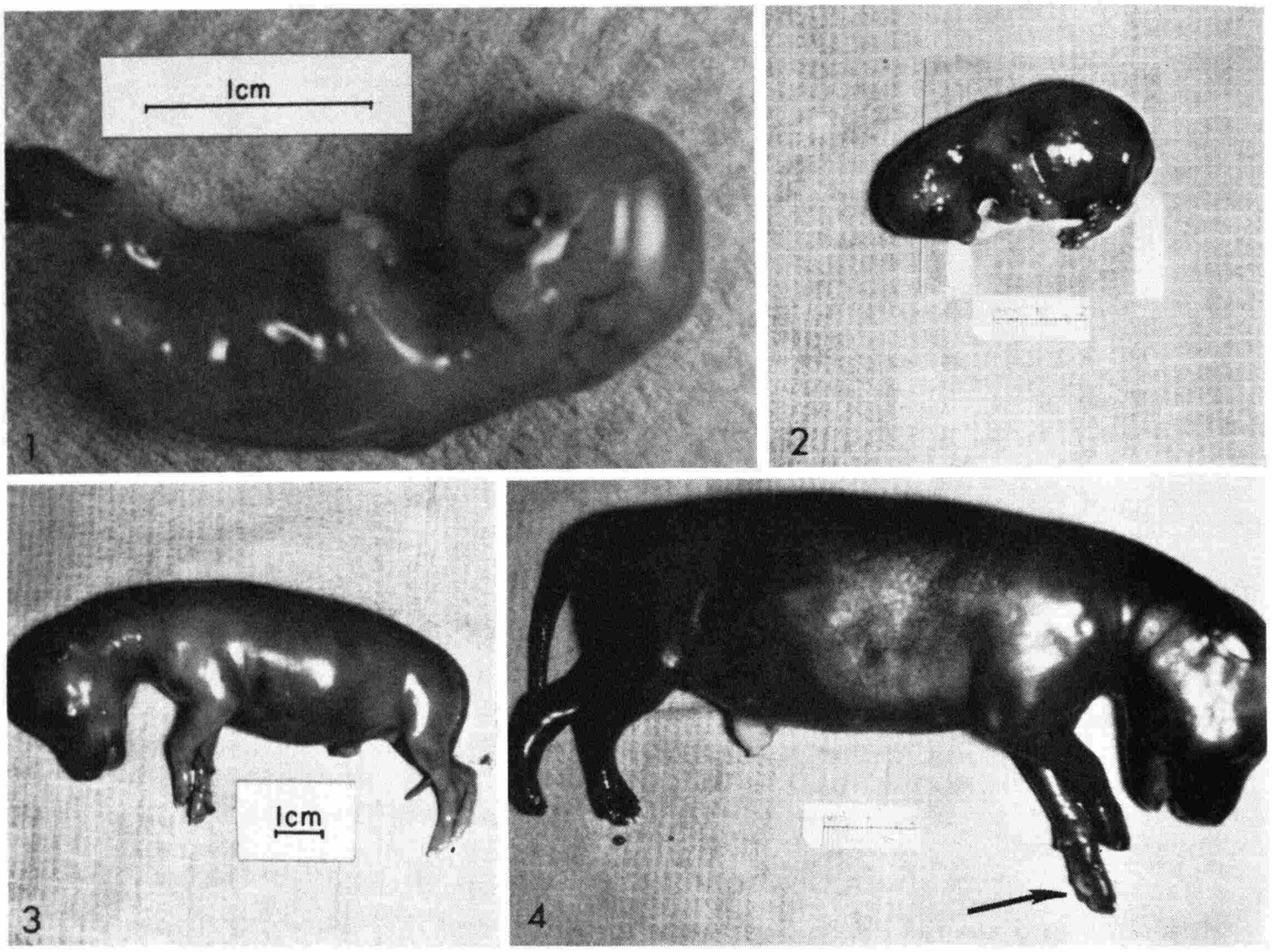

FIG. 1. Fetus, 29 days after breeding, 28 mm crown-rump length. Prominent retinal pigment. Earliest cutaneous melanocytes present but melanin not detectable grossly.

FIG. 2. Fetus, 37 days after breeding, 58 mm crown-rump length. Gross cutaneous pigmentation present, chiefly in the skin of muzzle, eyelids, and external ears.

FIG. 3. Fetus, 40 days after breeding, 98 mm crown-rump length. Gross cutaneous pigmentation is easily detectable in most areas.

FIG. 4. Fetus, 46 days after breeding, 160 mm crown-rump length. Abundant skin pigmentation present. Gross pigment not detected in claws and metacarpal pad (*arrow*). Body hair present.

the eyes and ears, the thorax, principally dorsally and medially, and to a lesser degree, the abdomen. It was not possible to distinguish between dermis and hypodermis, but melanoblasts were found in the lower two thirds of the dermis. Many were in contact with or near blood vessels (Fig. 6). Immature spindle-shaped melanocytes were occasionally observed in the mesenchyme of the posterior head, primordial eyelid, external ear, and dorsal thorax of 25 to 28 mm fetuses. Some were seen immediately beneath the covering epithelium (Fig. 7). The cells averaged 4 μ x 40 μ and contained numerous melanosomes. In 32 to 42 mm fetuses, melanoblasts in varying stages of maturation were observed in the mesenchyme of the abdomen, eyelids, external ear and thorax (Fig. 8). Limited numbers of melanoblasts characterized by faintly detectable melanin, were observed in untreated* tissues. In a 58 mm fetus, melanoblasts were seen in the mesenchyme of the abdomen, back, tail and eyelid. Earliest poly-dendritic dermal melanocytes were demonstrated in a 58 mm fetus. They were seen in dopa-treated, ASN-treated and untreated sections of the abdomen, back, eyelid, external ear, neck and tail. They were most abundant in the intermediate and outermost portions of the dermis. Dendritic melanocytes were not found in the dermis of abdomen, feet, scalp, lip, gum, hard palate and planum nasale.

* Untreated as used here and elsewhere in this report indicates that the tissue was not stained for melanin or premelanin.

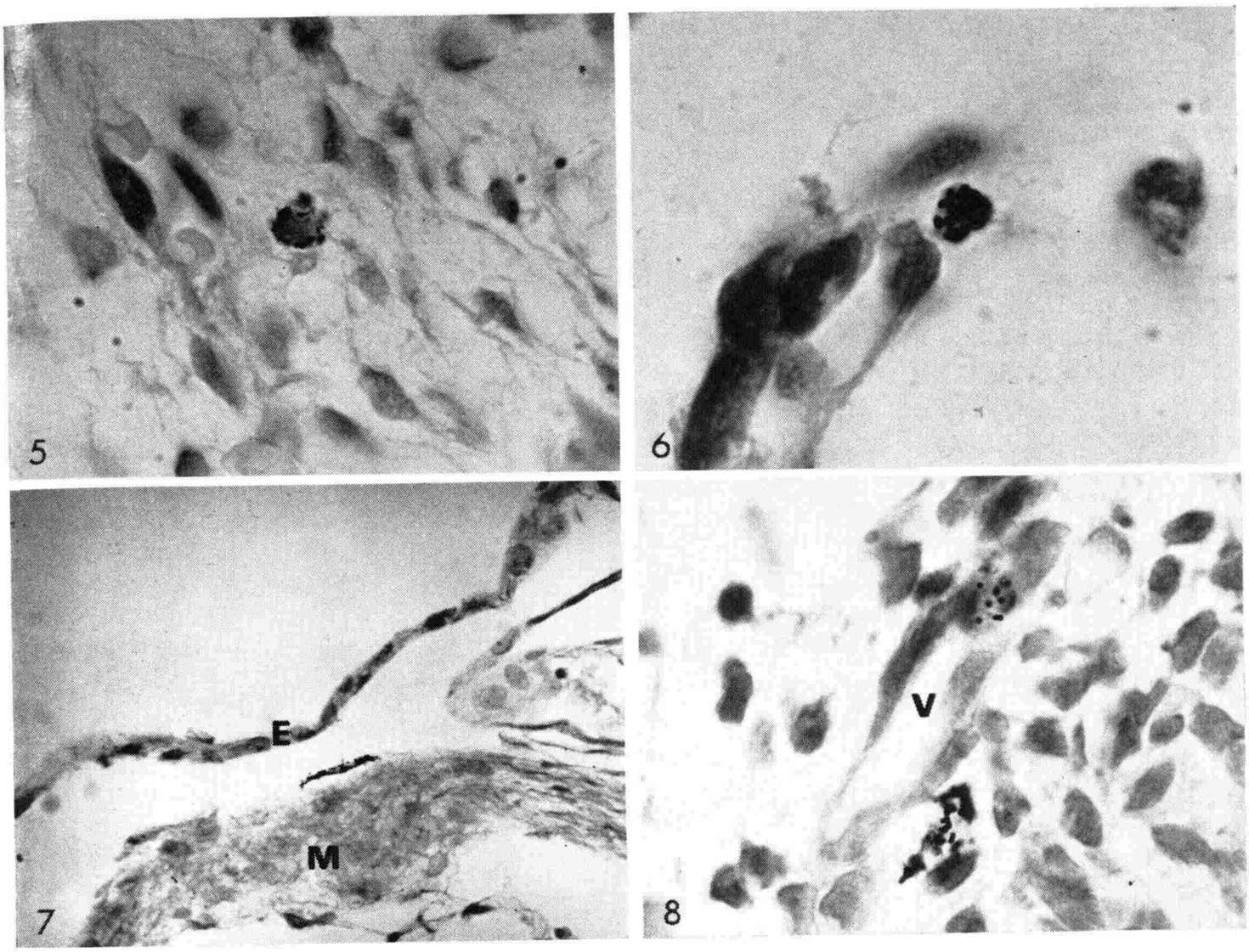

FIG. 5. Melanoblast in the fetal dermis of the dorsolateral thorax. 28 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 878.

FIG. 6. Melanoblast in contact with a blood vessel in the dermis of the neck; 28 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 2,400.

FIG. 7. Immature melanocyte between the epithelium (E) and mesenchyme (M). Dorsum of the head, 28 mm crown-rump length. Ammoniacal silver nitrate and toluidine blue. × 366.

FIG. 8. Early melanoblast (above) and one approaching maturation (below) in contact with a vessel (V) in the fetal dermis near the ear; 32 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 1,600.

In 98 to 160 mm fetuses, melanoblasts were occasionally demonstrated in ASN-treated sections of abdomen, neck, lip, scalp, tail and many in sections of eyelid. They were demonstrated in dopa-treated (Fig. 9) and ASN-treated as well as in untreated tissue (Fig. 10). A few melanoblasts were found in the dermis of the feet, eyelids, cutaneous surface of the lip, and tail of a 195 mm fetus. Melanocytes were occasionally demonstrated in dopa-treated, ASN-treated and untreated sections of abdomen, back, cutaneous part of the lip, eyelid, external ear, neck, and tail. They were not found in sections of digital and metapodial pads, scalp and lip. The distribution of melanoblasts and melanocytes observed in the fetal dermis is summarized in Table II.

2. *Epidermal melanocytes.* Epidermal melanocytes were first observed in 25 to 28 mm. fetuses. Epidermal melanocytes could be identified in untreated skin by the presence of faint brown melanin granules. Demonstration of melanocytes was facilitated by staining with ASN solution (Figs. 11 & 12), or dopa.

In 28 to 32 mm fetuses, epidermal melanocytes were demonstrated in the head, neck, thorax, abdomen, legs and tail. The distribution of pigment cells in the epidermis of the neck and trunk was similar to that observed in the head in that melanocytes occurred in greater numbers in dorsal and dorso-lateral regions than in ventral regions.

In 36 to 42 mm fetuses, the covering epithelium ranged from 1 to 4 cell layers with 1

to 2 layers present in dorsal body regions and 3 to 4 cell layers ventrally. Where multiple layers existed, epidermal melanocytes were observed in the outer, intermediate and inner strata. The density distribution of epidermal melanocytes suggested a posterior-anterior gradient in the head, a dorsoventral gradient in the neck and trunk, and a proximodistal gradient in the limbs.

The epidermis of 58 mm fetuses was from 3 to 6 cells thick in the sites examined. Melanocytes were most numerous in the second layer (suprabasal), while limited numbers were found in the innermost layer (basal) (Fig. 13). They were demonstrated in the epidermis of abdomen, back, eyelid, external ear, feet, neck, scalp, tail and cutaneous surface of the lip, but they were not found in the digital pads, the distal two-thirds of the claws, mucosal surface of the lip, the gum or hard palate.

Epidermal melanocytes were found in the suprabasal layer and to a lesser extent in the basal layer of 98 mm fetuses. They were present in the skin of the abdomen, back, eyelid, external ear and feet, including the claws and distal parts of the digital pads, scalp, neck, lip (transitional zone and cutaneous part), planum nasale and tail. None was observed in the gum or hard palate.

The epidermis of a 160 mm fetus averaged 3 to 5 cell layers in hair regions and 5 to 9 layers in the digital pads. Melanocytes were observed in about equal numbers in both the basal and suprabasal layers in all sites examined except for the digital pads and lip, where they were more abundant in the suprabasal layer. They were found in the epidermis of the abdomen, back, neck, eyelid, external ear, lip, scalp, tail and feet except for the metapodial pad and the hairy skin that lies between it and the digital pad. None was found in the mucosa of the gum or hard palate.

The epidermis of a 195 mm fetus averaged 3 to 5 cell layers in hairy regions and ranged from 12 to 24 layers in the pads of the feet. The distribution of epidermal melanocytes is summarized in Table III.

3. *Melanocytes of hair follicles and other skin adnexa.* The terms pre-germ, hair germ, hair peg and bulbous peg used by Pinkus (18) to designate the different developmental stages of human embryonic hair follicles were used in this investigation.

The earliest hair follicles were observed around the mouth of a 28 mm fetus. These were hair germ stages of tactile follicles and contained melanocytes demonstrable both in ASN treated and untreated sections. The earliest follicles of body hair were found along the

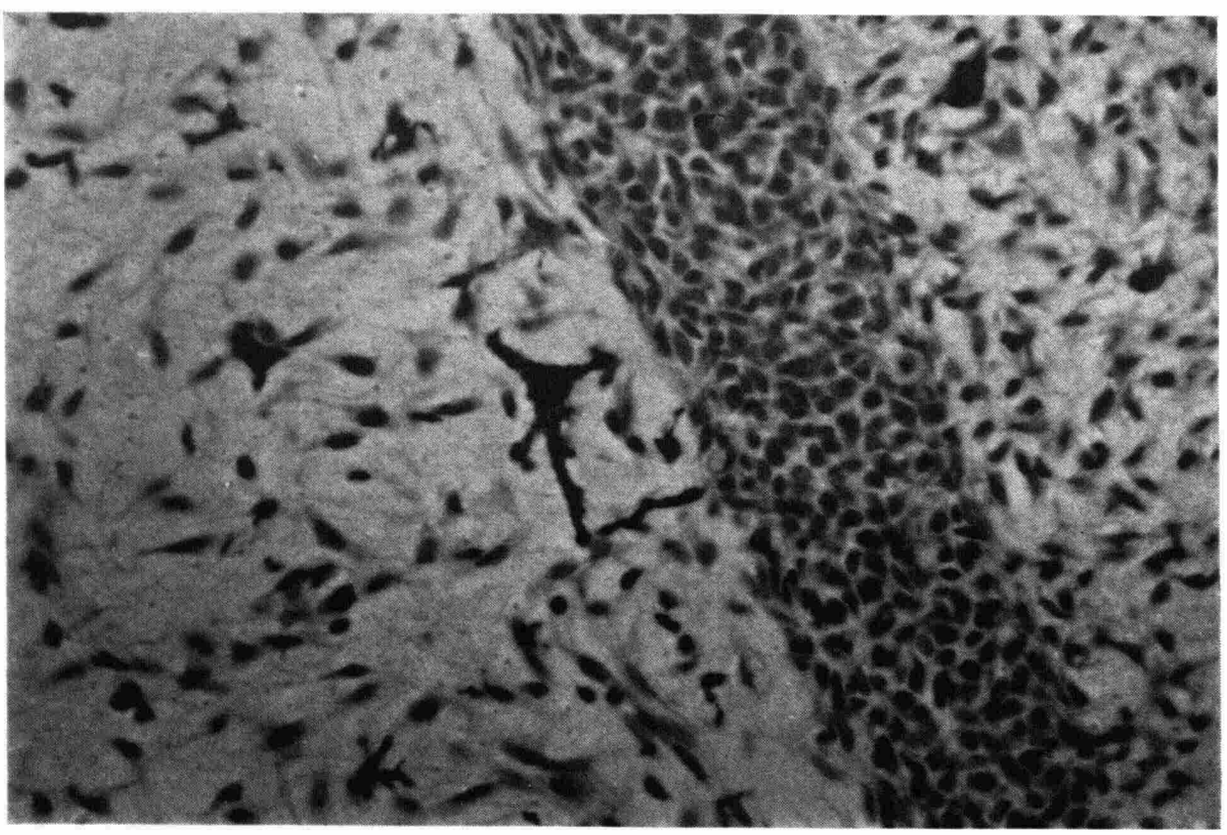

FIG. 9. Dopa-positive dermal melanocyte near the cartilage of the external ear; 98 mm crown-rump length. Dopa and brazilin. × 520.

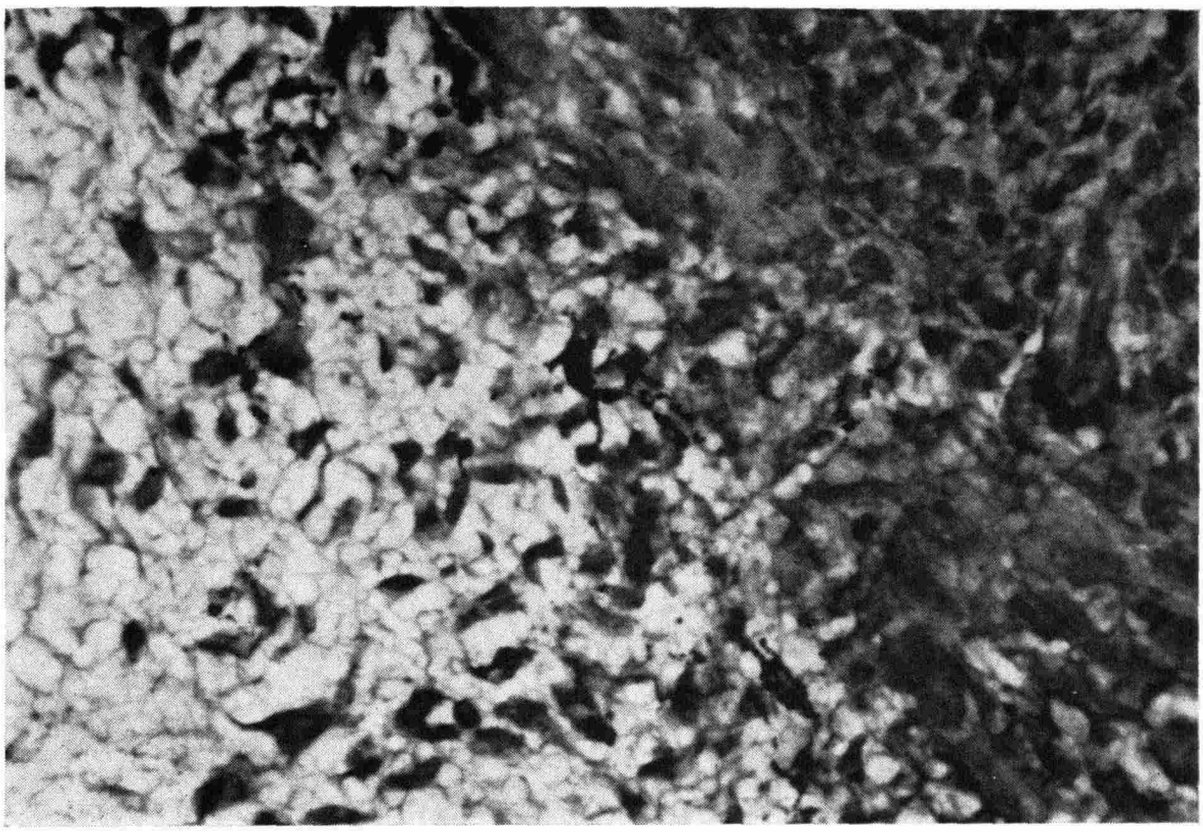

FIG. 10. Dermal melanocytes, unstained for melanin (arrows). External ear; 98 mm crown-rump length. Periodic acid-Schiff reaction. × 520.

dorsal and lateral surfaces of 32 mm fetuses, mostly in pre-germ stages (Fig. 14). Hair germ stages were occasionally observed in the skin of the head and neck of 32 mm fetuses.

In 36 to 42 mm fetuses, both pre-germ and hair germ stages were observed in the head, neck, thorax, abdomen, legs and tail.

Hair germs and hair pegs were found in the skin of the back, eyelid, neck, and scalp of 58 mm fetuses. Pre-germs or hair germs were found in the skin of the external ear, feet, lip, and tail. Pre-germs and early hair germs usually lacked melanocytes (Figs. 14 and 15) while melanocytes usually were present in older hair germs (Fig. 16) and hair pegs. Melanocytes were demonstrated in developing hair follicles of untreated, ASN-treated, and dopa-treated skin.

In 98 mm fetuses hair germ, hair peg and bulbous peg stages were prevalent; pre-germs were occasionally seen.

In 195 mm fetuses most primary cover hair follicles had developed to the bulbous peg stage. Melanocytes were most numerous in the hair bulb, but were also observed in the upper half of the hair follicle.

Melanocytes were not seen in the earliest anlagen of eccrine sweat glands which first were found in the pads of the feet of 98 mm fetuses. In 160 mm fetuses melanocytes were found in all parts of developing eccrine sweat glands, but only in those parts of the digital pads where the overlying epidermis contained melanocytes. In a 195 mm fetus, the eccrine glands consisted of the excretory duct and coiled secretory segment. Melanocytes were limited to the excretory duct and none was found in the deeper secretory portion.

Melanocytes and more commonly melanin were found among generative cells of sebaceous glands and occasionally in apocrine sweat glands in fetuses of 160 mm and larger.

DISCUSSION

Results of this study suggest that events leading to cutaneous pigmentation are as follows: (1) Melanoblasts originating from the neural crest (1–5) migrate along the adventitia of blood vessels in the mesenchyme. (2) Melanoblasts mature during migration. (3) Most melanocytes enter the epidermis while limited numbers remain in the dermis. (4) Some epithelial structures including skin adnexa, oral mucosa, the foot pads and the planum nasale are populated by melanocytes which migrate from adjacent pigmented sites.

There is adequate experimental evidence to support the hypothesis of neural crest origin of cutaneous melanocytes in vertebrates (1–5). DuShane (6) suggested that melanoblasts mi-

TABLE II

Distribution of melanoblasts and dermal melanocytes in Labrador retriever fetuses

Body regions	Crown-rump lengths of fetuses in mm					
	5–19	25–42	58	98	160	195
Abdomen	0	+	+	+	+	+
Back	0	+	+	+	+	+
Ear	0	+	+	+	+	+
Eyelid	0	+	+	+	+	+
Digital and metapodial pads	0	0	0	0	0	0
Gum	0	0	0	0	0	0
Hard palate	0	0	0	0	0	0
Lip	0	0	0	0	+[a]	+[a]
Neck	0	0	+	+	+	+
Planum nasale	0	0	0	0	0	0
Scalp	0	+	+	+	+	0
Tail	0	0	+	+	0	+

0 = Melanoblasts and melanocytes not observed.
+ = Melanoblasts or melanocytes observed.
[a] = Melanocytes observed in dermis of cutaneous portion only.

grate ventrolaterally, beneath the epidermis, however, the migratory pathways of melanoblasts have not been established. The frequent contact or close association of melanoblasts with blood vessels observed in this study suggested that melanoblasts migrate along the vascular adventitia in their journey from the neural crest to the skin.

The finding that earliest melanoblasts were observed only after tissue sections had been treated with ASN solution supports reports (15, 16, 19) that ASN can be used to demonstrate "pre-melanin" as well as melanin. Melanoblasts were not found in the untreated tissues of 25–28 mm fetuses or at earlier stages of gestation.

Our observations support the hypothesis of a dorso-ventral gradient for the appearance of melanoblasts in different loci.

In this study, round or ovoid cells containing faintly detectable melanin were demonstrated in the dermis of untreated sections of 32 to 195 mm. The presence of melanin in the cells suggested that melanogenesis was initiated while the melanoblasts were in transit and

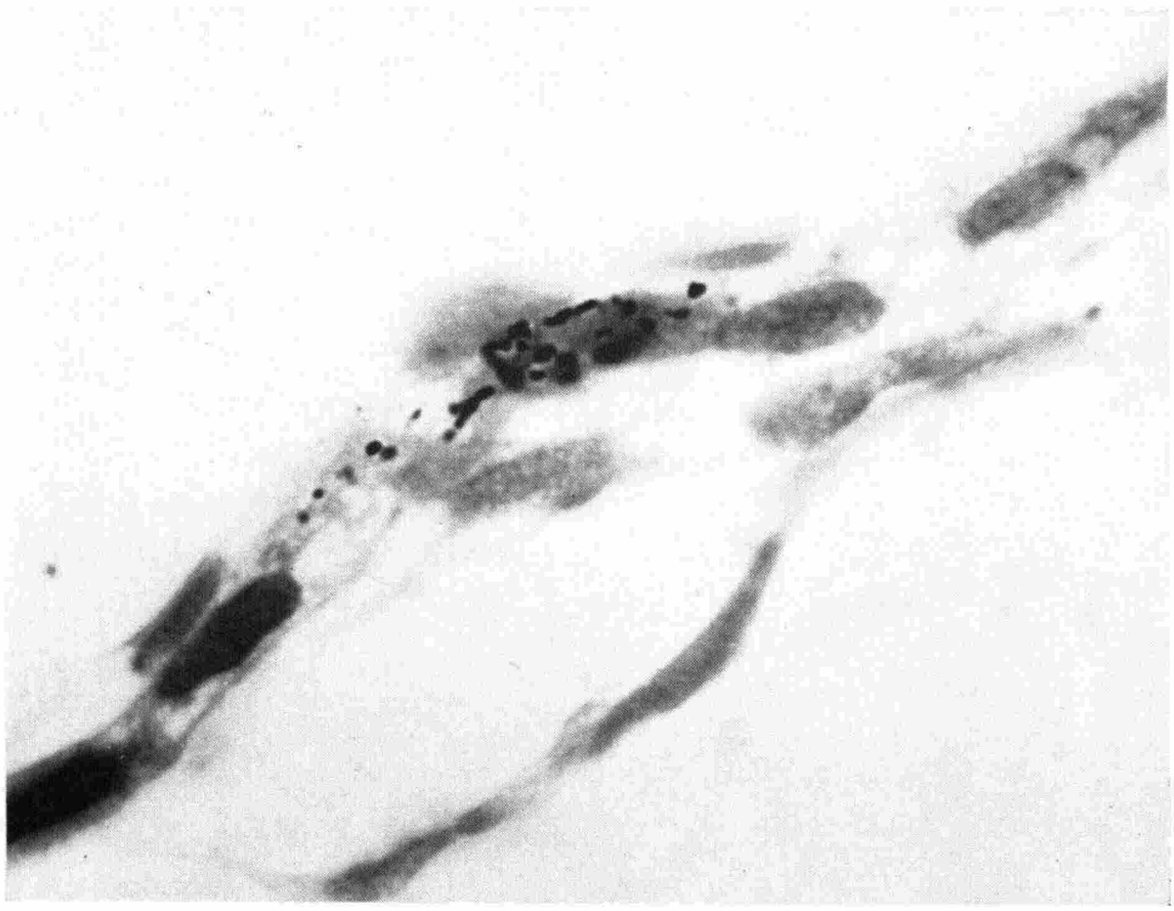

FIG. 11. Epidermal melanocyte from the neck; 28 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 1,600.

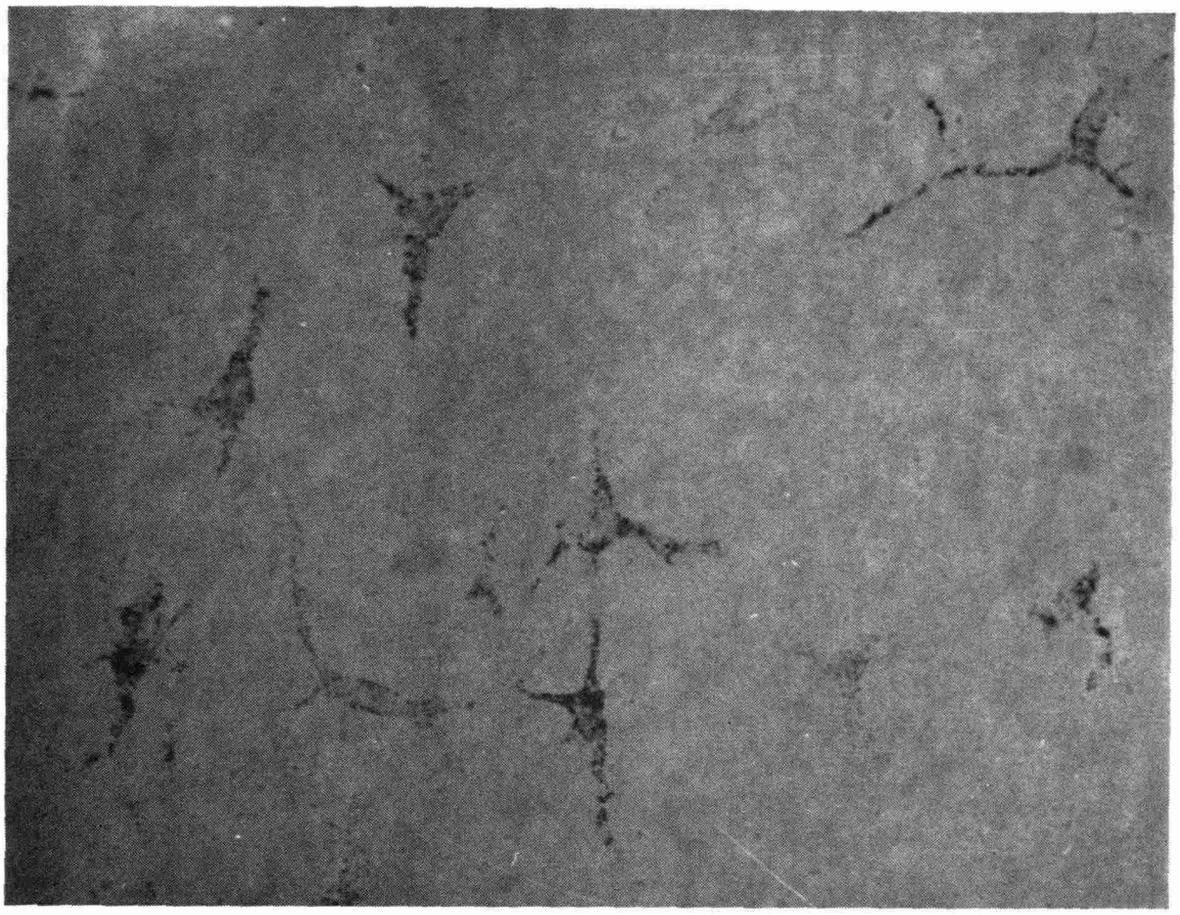

FIG. 12. Epidermal melanocytes in full thickness "spreads" from the dorso-lateral thorax of a fetus of 40 mm crown-rump length. Unstained (top). Ammoniacal silver nitrate (center). Dopa (bottom). × 512.

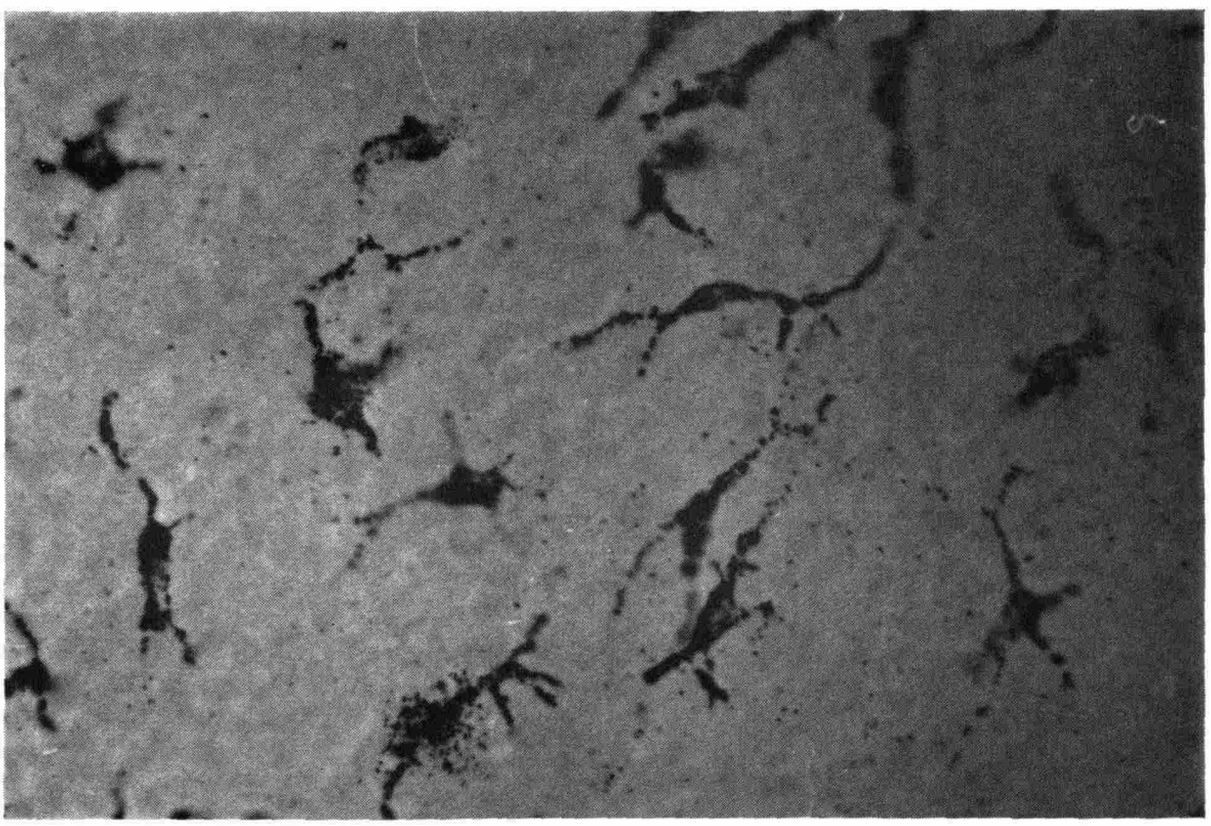

FIG. 12. (center)

prior to their departure from the vascular adventitia. Such cells were considered to represent melanoblasts of an advance stage of maturation. Although they contained limited amounts of melanin, they were comparable to non-melanin containing melanoblasts in size and structure.

Baker (13) did not find melanocytes in the

FIG. 12 (bottom)

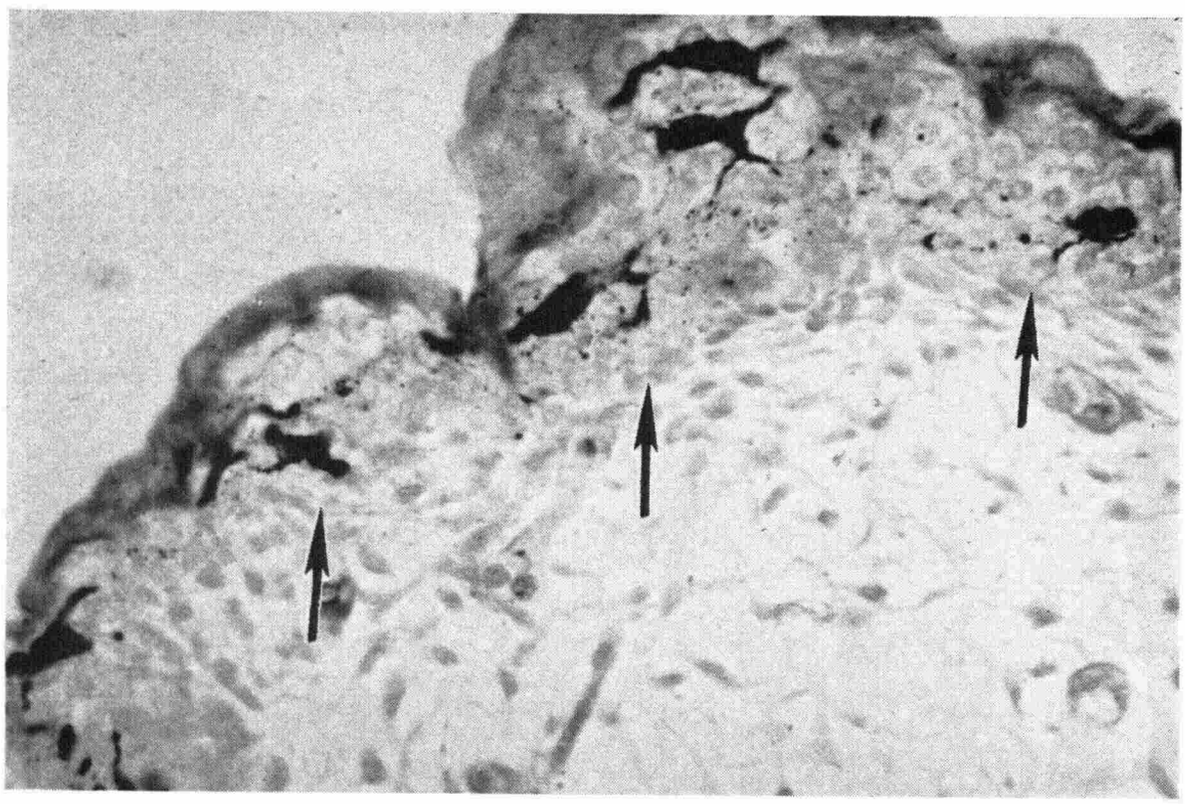

FIG. 13. Dopa-positive melanocytes in the basal and suprabasal layers of the epidermis. Neck, 58 mm crown-rump length. Dermal-epidermal junction (arrows). Dopa without counterstaining. × 520.

dermis of newborn pups. Similarly, Szabó (12) did not find melanocytes in the dermis of adult dog skin after treatment with dopa. However, in the present study, melanocytes were found in the dermis in all haired areas examined of an essentially full term Labrador retriever fetus. Melanocytes also have been observed in the dermis of abdomen, external ear, back, and eyelids of an 8 day old black pup of mixed breeding (20). In both instances, the pigment cells were demonstrated in skin unstained for melanin as well as in skin treated with ASN and dopa. It is clear that several questions remain unanswered: (1) do melanocytes persist in the dermis of adult dogs or (2) do they migrate into the overlying epidermis

TABLE III

Distribution of epidermal melanocytes in Labrador retriever fetuses

Body regions	Crown-rump lengths of fetuses in mm						
	5–19	25–32	36–42	58	98	160	195
Abdomen	0	+	+	+	+	+	+
Back (lumbosacral)	0	+	+	+	+	+	+
Ear	0	+	+	+	+	+	+
Eyelid	0	+	+	+	+	+	+
Feet							
Dorsum	0	0	0	+	+	+	+
Digital pads	0	0	0	0	0	+	+
Interdigital skin	0	0	0	0	0	0	+
Metapodial pads	0	0	0	0	0	0	+
Gum	0	0	0	0	0	0	0
Hard palate	0	0	0	0	0	0	0
Legs	0	+	+	+	+	+	+
Lip							
Mucosa	0	0	0	0	0	0	+
Transitional zone	0	0	0	0	+	+	+
Cutaneous	0	0	0	+	+	+	+
Neck	0	+	+	+	+	+	+
Planum nasale							
Peripheral part	0	0	0	+	+	+	+
Central part	0	0	0	0	0	0	+
Scalp	0	+	+	+	+	+	+
Tail	0	+	+	+	+	+	+

0 = Melanocytes not observed in epithelium.
+ = Melanocytes observed in epithelium.

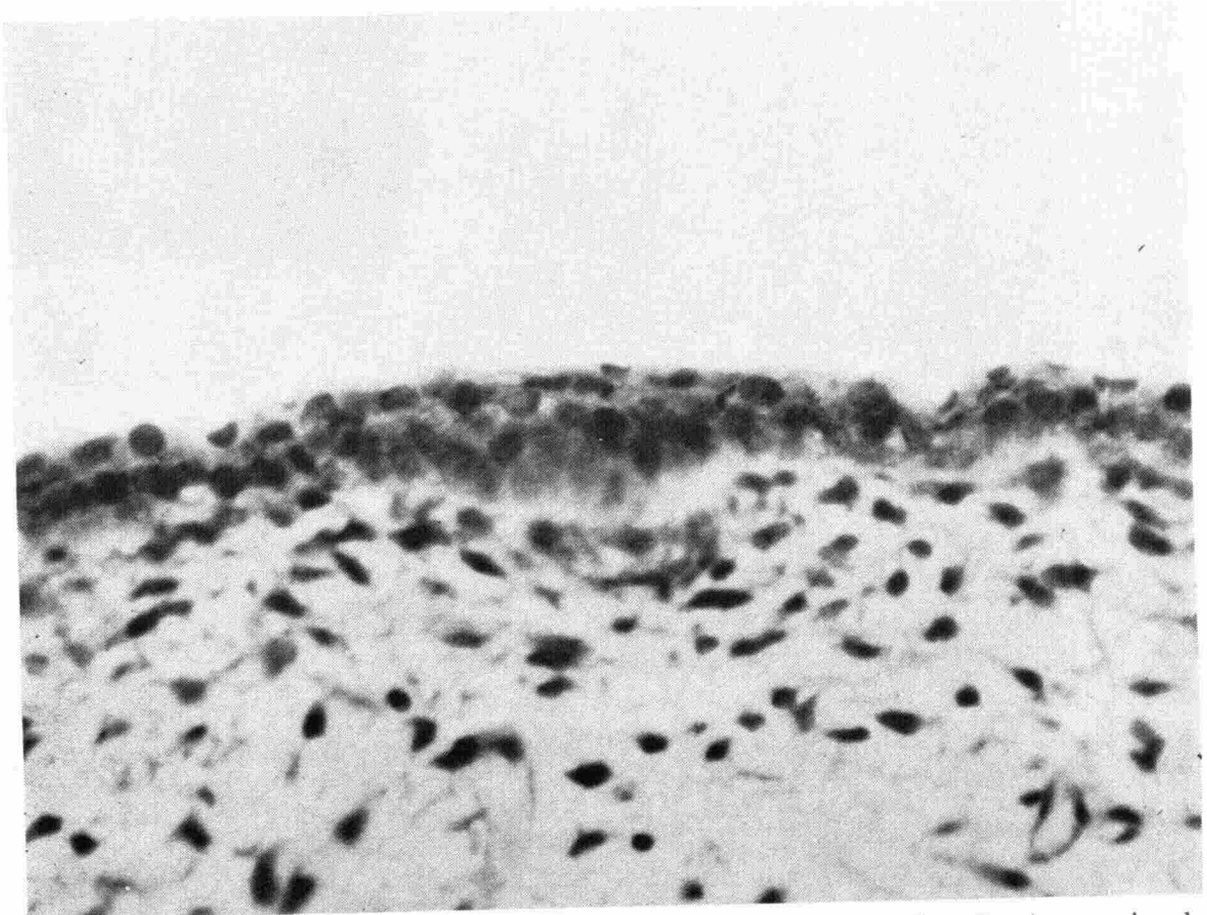

FIG. 14. Hair follicle pre-germ stage. Back, 32 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 435.

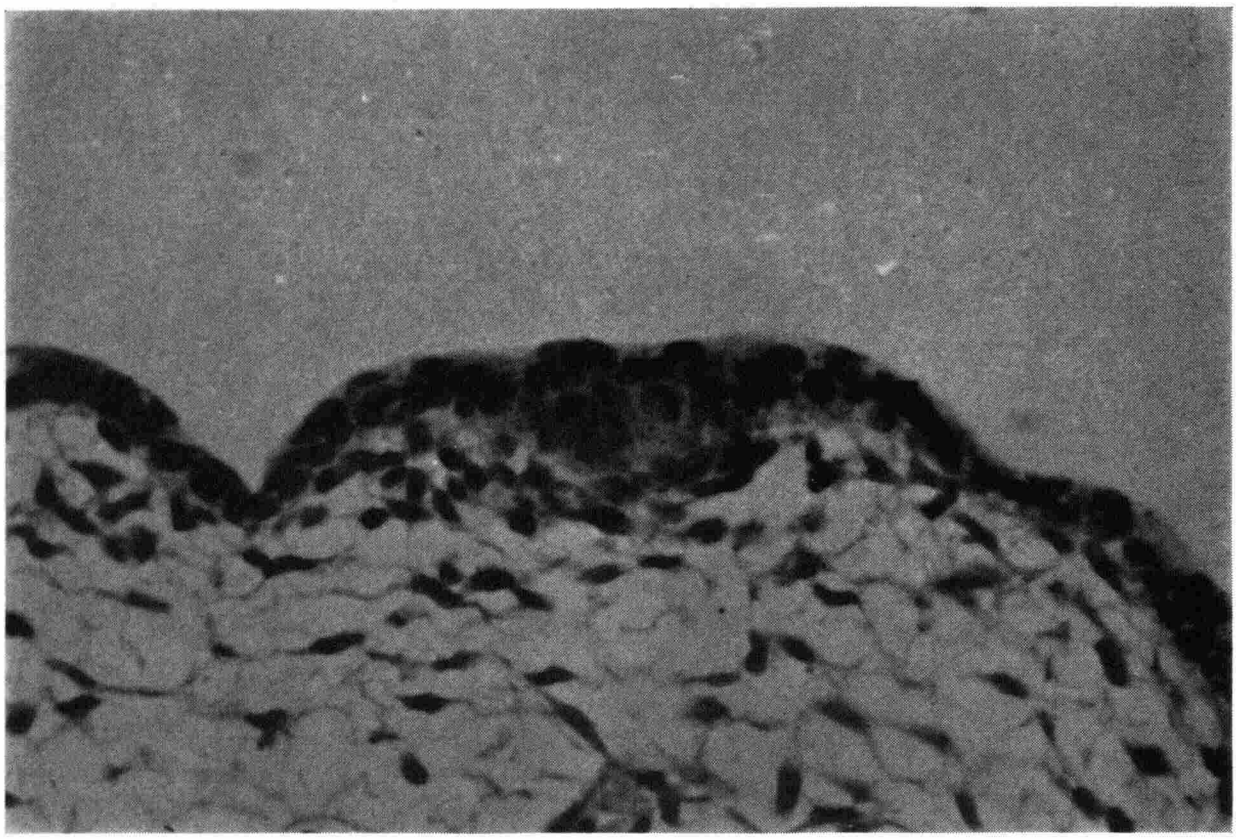

FIG. 15. Early hair germ stage with parts of melanocytes in the epidermis above and to one side of it. External ear, 58 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 512.

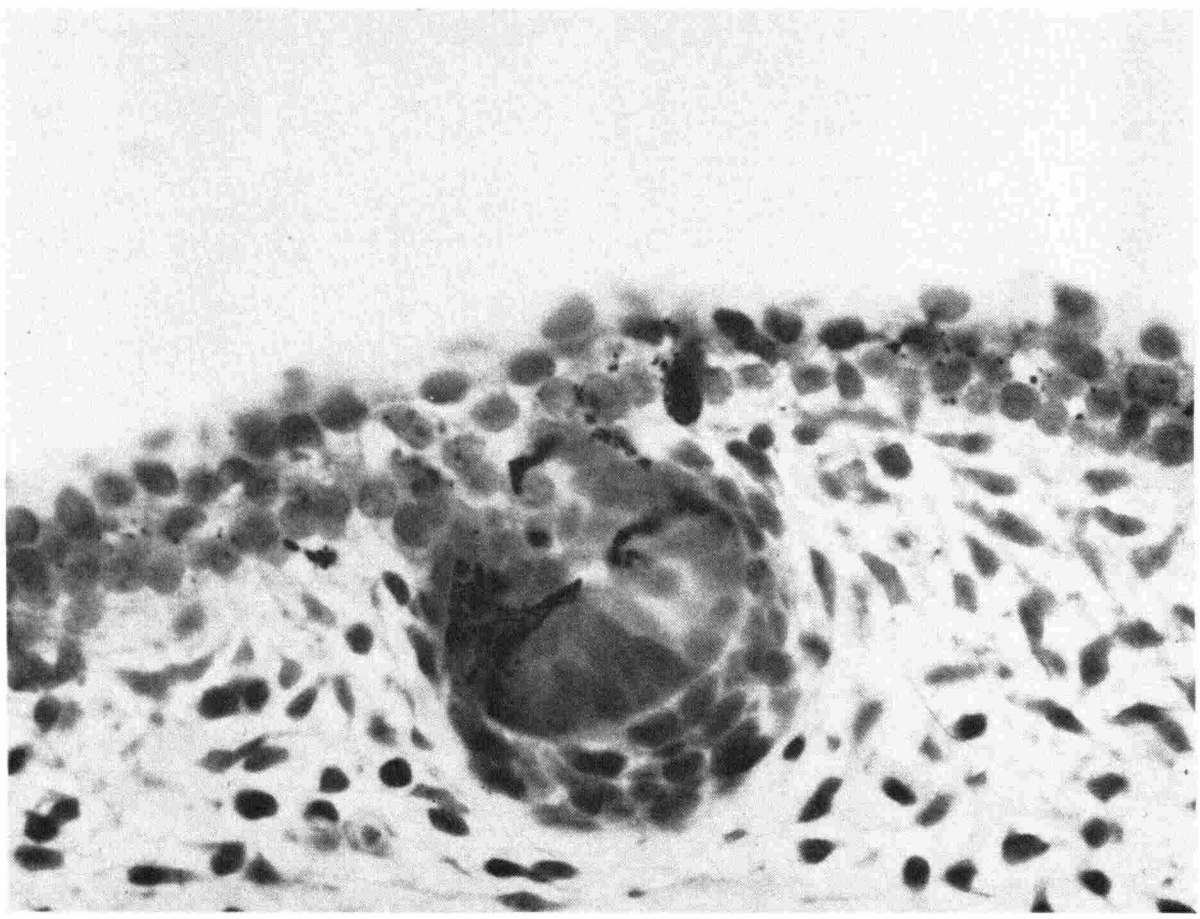

FIG. 16. Late hair germ stage containing melanocytes. Neck, 58 mm crown-rump length. Ammoniacal silver nitrate and brazilin. × 512.

some time after birth or (3) do they lose their capacity to form melanin, and therefore, remain undetected in the adult canine dermis?

Progressive pigmentation of digital and metapodial pads, mucosa of the lip, and planum nasale in the absence of demonstrable preexisting melanoblasts or melanocytes in the lamina propria or dermis suggests that melano-

cytes migrate within the epithelium to those sites from adjacent pigmented areas. However, the possibility that the epidermal melanocytes of these sites were derived from undetected melanoblasts was not completely excluded. The distribution and time of appearance of melanocytes in primordial hair follicles and eccrine sweat glands provide additional support for intraepithelial migration of epidermal melanocytes. Pre-germ and early hair germ stages of hair follicles usually did not contain melanocytes even though melanocytes were present in the adjacent epidermis. Melanocytes were chiefly found in later stages of hair follicle development, that is in more mature hair germs, hair pegs, and bulbous pegs. Similarly, primordial eccrine sweat ducts, represented by bulbous columns of cells, were present in the digital and metapodial pads prior to the occurrence of melanocytes in the covering epithelium of the pads. Melanocytes were demonstrated in upper portions of the sweat ducts only after melanocytes had been observed in the overlying epithelium. No attempt was made to determine the precise location of melanocytes in the developing hair follicles. Such a study would be desirable for comparison with similar studies of human hair follicle melanocytes (18, 21).

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